Overexpression of the Oxygen Sensors PHD-1, PHD-2, PHD-3, and FIH Is Associated with Tumor Aggressiveness in Pancreatic Endocrine Tumors

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Abstract

Purpose: Tumor hypoxia is associated with poor prognosis and resistance to treatment. Our aim was to assess the expression of proteins that act as cellular oxygen sensors, directly regulating the hypoxia inducible factor (HIF) pathway, i.e., prolyl hydroxylase domain proteins (PHD)-1, PHD-2, PHD-3, and FIH in pancreatic endocrine tumors (PET).

Experimental Design: Immunohistochemical expression of these markers was examined in 109 PET included in tissue microarrays and representing various stages of tumorigenesis. The results were correlated with histoprognostic factors including Ki-67 index, presence of a fibrotic focus, and microvascular density (MVD).

Results: The cytoplasmic and nuclear expressions of the three PHD isoforms were associated, and their expression was significantly higher in aggressive PETS, malignant, with lymph node metastases or with lower MVD. High nuclear expression of the three isoforms highly correlated with HIF-1α nuclear expression ($P = 0.02$, 0.003, and 0.006, respectively). Moreover, high nuclear PHD-1 or PHD-3 expression was associated with a poorer survival ($P = 0.01$). Cytoplasmic FIH was significantly higher in malignant PETs ($P = 0.05$) and in PETs with lymph node metastases ($P = 0.02$), and its expression correlated positively with those of cytoplasmic PHD isoforms ($P < 0001$). FIH stromal expression was found in 23% of PETs and correlated with higher FIH nuclear expression ($P = 0.0004$) and poorer disease-free survival ($P = 0.0018$).

Conclusion: HIF regulatory proteins are highly expressed in PET and their expression is correlated with tumor metastases, tumor recurrence, and prognosis. These molecules that play an important role in the control of hypoxia-induced genes may have a function in the regulation of cellular proliferation and differentiation during endocrine tumorigenesis.

Hypoxia and the hypoxia inducible factor (HIF)-1 pathway regulate the expression of a diverse group of genes that promote tumor growth and are involved in tissue invasion, angiogenesis, cell proliferation and survival, glycolysis, and pH regulation (1–3). Under conditions of hypoxia in most cancers, the HIF-1 pathway is activated, leading to up-regulation of many hypoxia-response proteins that are associated with an aggressive tumor phenotype (4–7). In pancreatic endocrine tumors (PET), the regulation of HIF signaling seems to be specific, HIF-1α and its downstream angiogenic molecule vascular endothelial growth factor being expressed at high levels in the cytoplasm of tumor cells in highly vascularized benign tumors (8).

Recent studies have shown that the HIF prolyl hydroxylases domain proteins (PHD-1, PHD-2, and PHD-3), together with the HIF asparaginyl hydroxylase factor–inhibiting HIF (FIH), act as cellular oxygen sensors, directly regulating the activity of the transcriptional complex HIF-1 that mediates the response to hypoxia, in response to changing oxygen levels. HIF-1 comprises a heterodimer of HIF-1α and HIF-1β. Degradation and inhibition of the limiting HIF-1α subunit are intimately connected in normoxia. Hydroxylation of proline residues by the three PHD isoforms earmarks the protein for proteasomal degradation by enabling its recognition by the von Hippel-Lindau tumor suppressor protein, whereas hydroxylation of an asparagine residue by FIH reduces its transcriptional activity (1, 9–12).

HIF-regulatory proteins have been shown to have a wide distribution in epithelial cells with very variable expression patterns (13, 14). This suggests that different HIF regulatory pathways may be operative in different tumor types. Given the importance of these molecules in the control of hypoxia-induced gene expression, our aim was to establish their expression pattern in a series of PETs and to correlate their level of expression with clinicopathologic characteristics including those that reflect intratumoral hypoxia such as presence of a fibrotic focus and angiogenesis.

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Materials and Methods

Patients and tumors. PETs were obtained from 109 patients who underwent pancreatic surgical resection at Beaujon Hospital between 1998 and 2006. Surgery consisted of distal pancreatectomy with splenectomy in 47 patients, pancreaticoduodenectomy in 37, and limited resection of the pancreatic body and/or neck (n = 21) or tail (n = 4). The clinicopathologic characteristics of the 109 cases are summarized in Table 1. For all cases, the diagnosis was established on routine formalin-fixed paraffin-embedded material. The histopathologic and clinical data recorded were as follows: age, sex, and presence of a clinical functional syndrome (insulinoma, glucagonoma, gastrinoma, somatostatinoma, and VIPoma). The tumors were classified in three groups according to the WHO 2000 criteria well-differentiated endocrine tumors, of benign prognosis, or of uncertain behavior and well-differentiated endocrine carcinomas (15, 16). As defined by this classification, well-differentiated tumors of benign or of uncertain behavior corresponded to nonmetastatic cases, whereas well-differentiated carcinomas corresponded to metastatic cases. No poorly differentiated carcinomas were analyzed. The tumors were also classified into stages (stage 1-4) and into grades (grade 1-3) according to the newly proposed tumor-node-metastasis (TNM) classification (17). The size of the tumors, presence of lymph node metastasis, and percentage of cell nuclei stained for Ki-67 (MIB1 antibody) were reported as well as presence of a fibrotic focus, defined as a scar-like fibrosclerotic area located within the tumor (18, 19). In accordance with previously reported studies, tumors with high proliferative index (>2%) were associated with greater tumor size (P = 0.0001), higher WHO disease stage (P < 0.0001), higher TNM grade (P = 0.0001) and stage (P = 0.0001), lower microvascular density (MVD; P = 0.007), necrosis (P = 0.0001), presence of liver (P = 0.003) or lymph node (P = 0.0013) metastases, and poorer survival (P = 0.005) and disease-free survival (P = 0.0001) in our series (8, 17, 20–23). Moreover, tumor grade and stage according to the newly proposed TNM classification correlated, respectively, with survival (P = 0.02 and P = 0.1; nonsignificant) and disease-free survival (P < 0.0001 and P = 0.0005).

Tissue array construction. Three tissue microarrays blocks were constructed from the 109 formalin-fixed, paraffin-embedded PET. One paraffin-embedded tissue block was selected for each tumor. The construction of these tissue microarrays was done using a tissue arrayer (Manual Tissue Arrayer-MTA1; Beecher Instruments, Inc.). The cores were taken at random inside the tumors, and each tumor specimen was represented by two 1-mm cores on the TMA.

Table 1. Patients demographics and main pathologic features

Characteristics	No. of patients; n = 109
Age (y)	
<40	22 (20%)
≥40	87 (80%)
Sex	
Male	47 (43%)
Female	62 (57%)
Von Hippel-Lindau disease	
Present	12 (11%)
Absent	97 (89%)
Multiple endocrine neoplasia type 1 disease	
Present	5 (5%)
Absent	104 (95%)
Functional syndrome	
Present	24 (22%)
Absent	85 (78%)
Size	
≤20 mm	37 (34%)
>20 mm	72 (66%)
Classification according to WHO 2000	
Benign endocrine tumors	31 (28%)
Endocrine tumors of uncertain behavior	27 (25%)
Well-differentiated endocrine carcinomas	51 (47%)
Staging according to TNM 2006*	
Stage 1	31(28%)
Stage 2	24 (22%)
Stage 3	28 (26%)
Stage 4	26 (24%)
Grading according to TNM 2006*	
Grade G1	41 (38%)
Grade G2	63 (58%)
Grade G3	5 (4%)
Necrosis	
No necrosis	97 (89%)
Presence of necrosis	12 (11%)
Ki-67 (%)	
≤2	55 (50%)
>2	54 (50%)
Lymph node metastasis	
Absent	67 (61%)
Present	42 (39%)
Liver metastasis	
Absent	83 (76%)
Present	26 (24%)
Duration of follow-up (mo)	
Median	22
Range	1-130
Lost to follow-up	7
Death	8

*Proposed classification, ref. 17.

Immunohistochemistry

Antibodies and immunohistochemical techniques. All the antibodies are reactive in paraffin-embedded sections. The PHD-1, PHD-2, PHD-3, FIH, HIF-1α, Ki-67 proteins, and CD34 antigen were detected using the following murine monoclonal antibodies 112, 76a, 188e, 162c, ESEE 122, MIB-1 (Dako), and QBEND10 (Immunotech), respectively (13, 14, and 24). Immunostaining of paraffin sections was done after dewaxing and rehydrating slides. Antigen retrieval was conducted by pretreatment with high temperature. Endogenous peroxidase was blocked with 0.5% hydrogen peroxide in water for 30 min. Substitution of the primary antibody with PBS was used as a negative control. To detect PHD-1, PHD-2, PHD-3, and FIH, respectively, antibodies were applied at 10 μg/mL and incubated at room temperature for 90 min. The Envision kit secondary antibody (Dako) was applied for 30 min and the peroxidase reaction was developed using diaminobenzidine provided in the kit. An automatized technique (Streptavidine-peroxidase with an automate Ventana; Benchmark) was used for the detection of Ki-67 and CD34.

Index of proliferation evaluation. Considering the heterogeneity in the distribution of the expression of Ki-67 in most endocrine tumors, the proliferation index was evaluated in a selected tumor block for each case and not in tissue microarrays. The proliferation index was calculated in an area of high Ki-67 staining, which was chosen at a low optical power (objective, ×10), in three high-power fields (objective, ×40) containing at least 2,000 cells. Tumors with ≤2% Ki-67 were considered at low proliferative index, whereas those with >2% Ki-67 were considered as having a high proliferative index according to WHO classification (15, 16).

MVD evaluation. The quantification of microvessel density was done after immunostaining with CD34 antibody at high optical power (objective, ×25). Vessels with a clearly defined lumen or well-defined linear vessel shape were taken into account for counting. The vessels were counted in each 1-mm diameter core and the core with the greater value was taken into account in each tumor. Tumors with a MVD

greater than the mean value (i.e., 372 microvessels/mm^2) were considered at high MVD, whereas those with ≤372 microvessels/mm^2 were considered at low microvessel density.

Scoring methods. For all the other antibodies tested (PHD-1, PHD-2, PHD-3, and FIH), immunohistochemical staining was evaluated in a semiquantitative fashion. A score was calculated, obtained by multiplying the intensity (negative scored as 0, weak scored as 1, moderate scored at 2, and strong scored as 3) by percentage of stained cells (0, no cells; 1, 1-10%; 2, 10-50%; 3, 50-75%; and 4, 75-100%). The mean score of the two cores that represented each tumor was taken into account. A median score was calculated for each protein and tumors with a score greater than the median value were considered at high protein expression. The staining of the stroma and of the tumor microvessels were also recorded, as negative or positive. Tumors with no vessel or no stroma stained on both cores were considered as negative.

Intraobserver and interobserver variability

Because the semiquantitative assessment of the proteins tested (PHD-1, PHD-2, PHD-3, and FIH) is in part subjective, 1 core taken at random in 20 patients were selected to calculate intraobserver variability. The second analysis for intraobserver variability was done 15 d after the first one. The assessment of the interobserver variability was calculated using the κ test.

Statistical analysis

General characteristics were expressed as median and range or percentage. Differences were assessed using the Kruskall-Wallis test for continuous data and the χ^2 test or the Fisher's exact test for categorical data, as categorical tumor variables [WHO classification, TNM grading and staging, tumor size (>2 cm considered as large according to the WHO classification)], presence of lymph node and/or liver metastasis, Ki-67 proliferation index (≤2% Ki-67 considered as low), MVD (≤372 microvessels/mm^2 considered as low), and immunoreactivity with the markers tested (tumors with a score greater than the mean value considered as positive). Moreover, the Pearson Product Moment correlation coefficient (PMCC) was used to assess the independent significance of the four markers PHD-1, PHD-2, PHD-3, and FIH using semiquantitative means.

Survival was calculated from the date of surgery. The end point of the follow-up period was February 2007. Seven patients were lost to follow-up. The main survival data are summarized in Table 1. Survival analyses for censored data were done using the Kaplan-Meier method for overall and disease-free survival. The Logrank test was used to compare survival data. Data were analyzed with the SAS 9.1 statistical software for Windows (SAS Institute, Inc.). All statistical tests were two-sided. The critical level of statistical significance was set at a P value of <0.05.

Results

Imunohistochemical analysis

PHD-1 expression. The cytoplasmic and nuclear scores ranged from 0 to 12 (median, 4; mean, 4.44) and from 0 to 4 (median, 0; mean, 0.63), respectively (Fig. 1A). Fifty-seven percent and 44% of the tumors were considered at higher cytoplasmic or nuclear expression, respectively. There was no expression of PHD-1 on stroma or vessel. PHD-1 cytoplasmic expression correlated positively with higher tumor size (P = 0.05), TNM grade (P = 0.05), higher Ki-67 index (P = 0.01), and lower MVD (P = 0.03). PHD-1 nuclear expression correlated positively with WHO disease stage (P = 0.01), higher TNM stage (P = 0.05), and presence of a fibrotic focus (P = 0.04). High nuclear PHD-1 expression was associated with poorer overall and disease-free survival (P = 0.01; Fig. 2).

PHD-2 expression. The cytoplasmic and nuclear scores ranged from 0 to 12 (median, 1.5, mean: 2.65) and from 0 to 5 (median, 0; mean, 0.39), respectively (Fig. 1B). Fifty-three percent and 30% of tumors were considered at higher cytoplasmic or nuclear expression, respectively. There was no stroma or vessel expression. Higher cytoplasmic expression of PHD2 was associated with malignant PET (P = 0.03). The nuclear expression of PHD2 was associated with malignant PET (P = 0.004), greater tumor size (P = 0.0001), TNM grade (P = 0.009) and stage (P = 0.04), higher Ki-67 index (P = 0.01), presence of lymph node metastases (P = 0.03), presence of a fibrotic focus (P = 0.04), and with higher rate of recurrence (P = 0.03) and death (P = 0.05).

PHD-3 expression. The cytoplasmic and nuclear scores ranged from 0 to 12 (median, 1.25; mean, 2.16) and from 0 to 4.5 (median, 0; mean, 0.55), respectively (Fig. 1C). Fifty-two percent and 39% of tumors were considered at higher cytoplasmic or nuclear expression, respectively. There was no stroma or vessel expression.

The cytoplasmic expression of PHD-3 was associated with malignant PET (P = 0.004), greater tumor size (P = 0.007), lower MVD (P = 0.04), and higher TNM grade (P = 0.01). PHD-3 nuclear expression correlated positively with WHO disease stage (P = 0.05), TNM grade (P = 0.03), and presence of a fibrotic focus (P = 0.04). Higher nuclear PHD-3 expression was associated with a higher Ki-67 index (P = 0.005) and with a poorer overall and disease-free survival (P = 0.01).

FIH expression. The cytoplasmic and nuclear scores ranged from 1 to 12 (median, 8; mean, 7) and from 0 to 9 (median, 1; mean, 1.84), respectively (Fig. 1D). Fifty-one percent and 61% of tumors were considered at higher cytoplasmic or nuclear expression, respectively. Cytoplasmic FIH expression was significantly higher in malignant PETs compared with well-differentiated tumors of benign or uncertain prognosis (P = 0.05) and in PETs with lymph node metastases (P = 0.02),

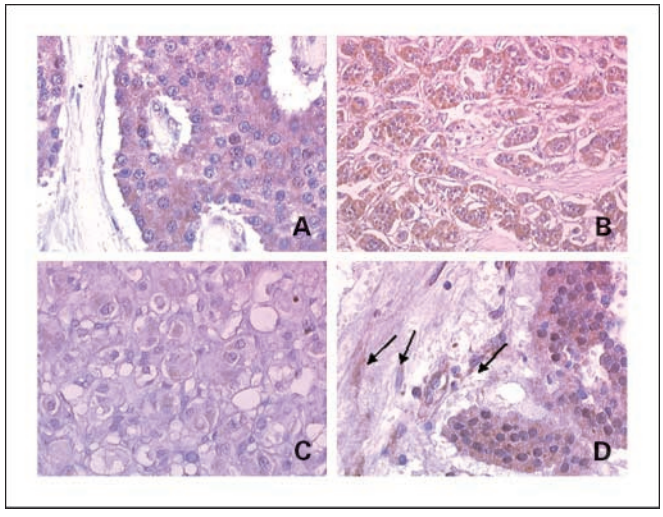

Fig. 1. Immunohistochemical expression of PHD-1 (*A*), PHD-2 (*B*), PHD-3 (*C*), and FIH (*D*) by pancreatic endocrine tumors. *A,* PHD-1 cytoplasmic expression is diffuse and moderate and nuclear expression is faint in 10% of tumor cells. *B,* PDH-2 cytoplasmic expression is moderate in 100% of tumor cells. *C,* PHD-3 expression in faint in 60% of the tumor cells. *D,* cytoplasmic expression of FIH is faint in all tumor cells, whereas nuclear expression is moderate in 50% of tumor cells. Fibroblasts in the stroma are positive.

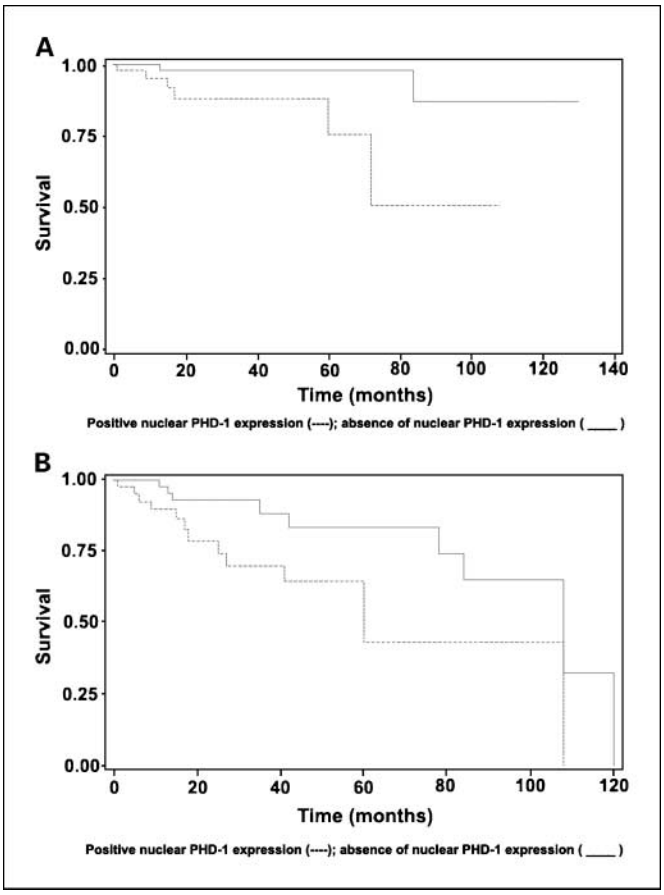

Fig. 2. Overall (*A*) and disease-free (*B*) survival curves according to Kaplan-Meyer according to nuclear PHD-1 expression (- - -) or absence of nuclear PHD1 expression (—); $P = 0.01$.

of greater size ($P = 0.017$), TNM grade ($P = 0.05$), and stage ($P = 0.02$). FIH stromal expression was found in 23% of PETs and correlated with poorer disease-free survival ($P = 0.0018$; Figs. 1F and 3). Nuclear FIH did not correlate with any histopathologic variable.

Correlations between PHD-1, PHD-2, PHD-3, and FIH. Using the Pearson's correlation test, we found positive correlations between the oxygen sensors. We found a relationship between cytoplasmic expression of the four markers [PHD-1 and PHD-2 (PMCC, 0.27; $P = 0.004$), PHD-1 and PHD-3 (PMCC, 0.34; $P = 0.0005$), PHD-2 and PHD-3 (PMCC, 0.48; $P < 0.0001$), FIH and PHD-1 (PMCC, 0.42; $P < 0.0001$), FIH and PHD-2 (PMCC, 0.49; $P < 0.0001$), and FIH and PHD-3 (PMCC, 0.47; $P < 0.0001$)]. We found a relationship between nuclear and cytoplasmic expression of FIH (PMCC, 0.28; $P = 0.003$), PHD-1 (PMCC, 0.26; $P = 0.006$), PHD-2 (PMCC, 0.17; $P = 0.05$), and PHD-3 (PMCC, 0.5; $P < 0.0001$). We found a relationship between nuclear expression of PHD-1 and PHD-2 (PMCC, 0.21; $P = 0.02$), PHD-1 and PHD-3 (PMCC, 0.38; $P = <0.0001$), and PHD-2 and PHD-3 (PMCC, 0.36; $P < 0.0001$).

HIF-1α expression. The cytoplasmic and nuclear scores ranged from 0 to 12 (median, 4.5; mean, 4.9) and from 0 to 4 (median, 0; mean, 0.12), respectively. Forty-nine percent and 37% of tumors were considered at higher cytoplasmic or nuclear expression, respectively. Higher cytoplasmic HIF-1α expression correlated with higher rate of disease-free survival ($P = 0.02$) and lower index of proliferation ($P = 0.03$). Higher nuclear HIF-1α expression correlated with higher nuclear expression of PHD-1 ($P = 0.02$), PHD-2 ($P = 0.003$), and PDH-3 ($P = 0.006$).

Intraobserver and interobserver variability for PHD-1, PHD-2, PHD-3, and FIH. Twenty cores in 20 patients were selected to perform this analysis. The intraobserver concordance was 90% [95% confidence interval (95CI%), 0.71-1.00] for nuclear FIH, 86% (95CI%, 0.59-1.00) for cytoplasmic FIH, 76% (95CI%, 0.54-1.00) for nuclear PHD-1, 78% (95CI%, 0.5-1.00) for cytoplasmic PHD-1, 68% (95CI%, 0.36-1.00) for nuclear PHD-2, 100% (95CI%, 1.00-1.00) for cytoplasmic PHD-2, 69% (95CI%, 0.28-1.00) for nuclear PHD-3, and 100% (95CI%, 1.00-1.00) for cytoplasmic PHD-3.

Microvessel counting. The microvessel density ranged from 40 to 1,243 vessels/mm^2 (median, 372; mean, 412). The MVD decreased with disease progression according to WHO classification ($P = 0.0004$). The MVD was lower in tumors of greater size ($P = 0.034$), TNM grade ($P = 0.0001$), and stage ($P = 0.0019$), with a high proliferative index ($P = 0.007$) and presence of lymph node metastases ($P = 0.003$). Low MVD was significantly associated with shorter survival ($P = 0.003$).

Fibrotic focus. Sixteen percent of tumors presented a fibrotic focus. The presence of a fibrotic focus correlated positively with disease progression according to WHO classification ($P = 0.007$) or TNM grade ($P = 0.03$) and stage ($P = 0.05$), with higher tumor size ($P = 0.01$) and the presence of lymph node metastases ($P = 0.03$).

Necrosis. Eleven percent of tumors presented necrosis. The presence of necrosis correlated with presence of a fibrotic focus ($P = 0.03$), higher TNM stage ($P = 0.008$) or grade ($P < 0.00001$), higher tumor size ($P = 0.0076$), and higher Ki-67 index ($P < 0.0001$).

Discussion

In this study, we used the tissue microarray technique to evaluate the expression of a panel of proteins that play a role

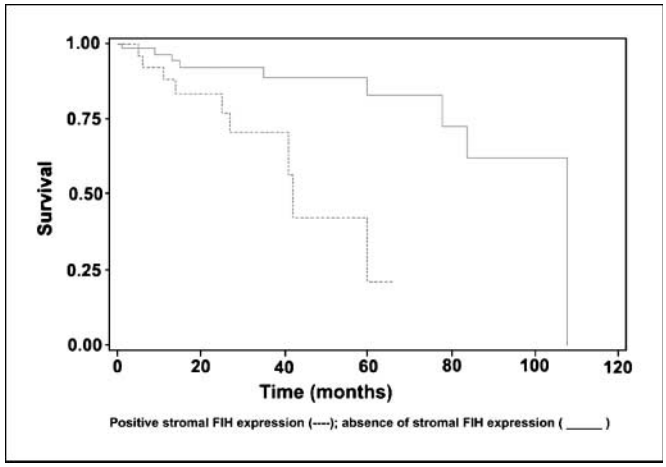

Fig. 3. Disease-free survival curves according to Kaplan-Meyer according to stromal FIH expression (- - -) or absence of stromal FIH expression (—); $P = 0.0018$.

in hypoxia regulation in pancreatic endocrine tumors. Hypoxia is a common feature of human cancers, associated with aggressive behavior, metastasis, and lower survival (6). Pancreatic endocrine tumors are highly vascularized and present specific regulation of the HIF pathway (8, 23). The best characterized molecular responses to hypoxia are mediated through the HIF-1 and HIF-2 transcription factor complex (6, 7). Under normoxic conditions, the regulatory α-subunit (HIF-1α) is posttranslationally hydroxylated at two proline residues (Pro^{402} and Pro^{564}; refs. 25–27). These are recognized by the von Hippel-Lindau tumor suppressor proteins that subsequently lead to ubiquitination and proteosomal destruction of HIF-1 (28). Three human prolyl hydroxylases that use oxygen as a cosubstrate have been characterized (29, 30) and termed prolyl hydroxylase domain proteins (PHD-1, PHD-2, and PHD3). Under restricted oxygen availability, the PHD activity decreases and the degradation of HIF-1α is blocked, leading to the transcription of a wide range of genes that have key functions in glucose homeostasis and angiogenesis. The three PHD isoforms are widely expressed in tissues; PHD-2 inhibition by RNA interference in human cells of different origin, but not inhibition of PHD-1 or PHD-3, is sufficient to up-regulate HIF-1α in normoxia, indicating that PHD2 may be the main cellular oxygen sensor (12–14, 31, 32). Interestingly, we found that a fibrotic focus, which reflects intratumoral hypoxia in many tumor types and is associated with PET aggressiveness in our series, is significantly associated with higher nuclear PHD-2 expression (19). Recently, Jokilehto et al. (33) have studied the expression of PHD2 in head and neck squamous cell carcinomas. They showed that expression and nuclear translocalization of PHD2 was associated with less-differentiated and strongly proliferating carcinomas. Our results showing that the nuclear expression of PHD2 is increased in aggressive PETs with higher grade and stage according to the newly proposed TNM classification and with higher rate of recurrence ($P = 0.03$) and death ($P = 0.05$) are in accordance with these data (17). These results suggest that the altered expression of PHD2 may depend on growth-promoting events and that PHD2 may have a function in the regulation of cellular proliferation and differentiation in PETs. Hypoxia is probably not the only trigger of altered PHD expression. This is confirmed by the results of Kato et al. (34), demonstrating PHD1 mutations in 60% of endometrial cancers.

The function of PHD2 is known to keep HIF-1α at a low level in normoxic conditions. On the other hand, expression of nuclear HIF-1α is a marker of poor prognosis in most carcinomas, including PET (8). Therefore, one might argue that elevated PHD2 expression is not in accordance with the known association of HIF in tumors because we identified an association between nuclear PHD2 and HIF-1α expression ($P = 0.004$). Our results suggest that the elevated PHD2 is not sufficient to down-regulate HIF-1α in PET. Jokilehto et al. (33) found similar results in head and neck squamous cell carcinomas because they identified tumor regions where simultaneous HIF-1α and PHD2 expression could be detected. These paradoxical results can be partly explained by the demonstration that HIF and PHDs form a feedback loop that limits hypoxic signaling, PHD2 being a direct HIF target gene (35, 36). Moreover, it is also possible that other cell types such as macrophages, which do not express detectable levels of regulatory enzymes by immunohistochemistry but were found to express a high level of HIF2α in a previous study (8), represent a way to activate the HIF pathway in tumorigenesis of PETs.

Interestingly, we showed a correlation of expression between the three PHD isoforms, both in the cytoplasm and in the nucleus of endocrine tumor cells. As for PHD2, the cytoplasmic and nuclear expression of PHD1 and PHD3 are correlated with more aggressive, malignant PETs. Their cytoplasmic expression is also associated with lower MVD, which is a hallmark of agressivity in PETs suggesting that PHD molecules play a role in the regulation of tumor angiogenesis through hypoxia signal and HIF regulation (8). In addition, we show that PHD1 and PHD3 nuclear expression is associated with a shorter survival in PETs ($P = 0.01$). These results are in accordance with those of Soilleux et al. (14) who show extremely variable expression of PHD isoforms between the cases of the same type of neoplasm they studied, probably reflecting differences among tumors with several malignant potential. Our results point out that all three PHD oxygen sensors play a role in the tumorigenesis of PETs.

The FIH hydroxylates the protein HIF-1α at asparagine 803, suppressing its interaction with transcription coactivators and reducing the transcriptional activity of the protein. One important substrate requirement for any enzyme is colocalization. The localization of HIF-1α, the substrate of FIH, is very specific in PETs because it is highly expressed in the cytoplasm of tumor cells in well-differentiated tumors and translocates to the nucleus in aggressive poorly differentiated ones (8). Using immunohistochemistry, Linke et al. (37) showed that FIH-1 is primarily confined to the cytoplasm under normoxia *in vitro* and does not translocate to the nucleus with HIF-α substrates under hypoxic conditions. This cytoplasmic localization is in agreement with that reported for osteosarcoma cells under normoxic and hypoxic conditions (35). Our study shows that, as for PHDs, the detection FIH predominates in the cytoplasm of tumor cells compared with the nucleus and is correlated with a malignant behavior and cytoplasmic expression of PHD1, PHD2, and PHD3. This colocalization supports the key role of the four oxygen sensors, including FIH, to target the cytoplasmic protein HIF-α for proteasomal degradation in aggressive PETs. Interestingly, we noted a positivity of FIH in the stromal cell of some tumors, mainly the fibroblasts, correlated with a lower disease-free survival, indicating the effect of the interaction between microenvironnement and tumor cells in the regulation of HIF signaling.

In conclusion, this study shows that HIF regulatory proteins are highly expressed in PETs both in tumor cells and in cellular elements of the stroma, and that their expression is correlated with tumor prognosis. This highlights the key role of these molecules, which play an important role in the control of hypoxia-induced genes, in the regulation of endocrine tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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